

ANALYSIS OF INDOLE COMPOUNDS IN *ARMILLARIA MELLEAE* FRUITING BODIES

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Abstract: A TLC method with densitometric detection for identification and quantitation of indole compounds in methanolic extracts of fruiting bodies of *Armillaria mellea* (Vahl.) P. Kumm. (*Basidiomycota*) – Honey mushroom has been developed. The TLC method with densitometric detection was validated for determination of tryptamine and serotonin contents. The use of this chromatographic-densitometric method for analysis of extracts allowed for a precise, easy and quick determination of the compounds under study. We identified three physiologically active indole compounds: tryptamine, L-tryptophan and serotonin. This analysis demonstrated the highest contents of L-tryptophan (4.467 mg/100 g d.w.). The contents of tryptamine were comparable with the contents of serotonin and amounted to: 2.740 and 2.207 mg/100 g d. w.

Keywords: tryptamine, tryptophan, serotonin, methanolic extract, *Armillaria mellea* fruiting bodies, TLC with densitometric detection

Our earlier mycochemical studies of fruiting bodies from the selected representatives of higher fungi belonging to the largest taxon *Basidiomycota* have demonstrated that they contain many nonhallucinogenic indole compounds, like L-tryptophan, tryptamine, 5-hydroxytryptophan and serotonin (1, 2). The indole compounds were identified in fruiting bodies of the conditionally edible species *Lactarius deterrimus* (1) as well as in fruiting bodies and *in vitro* cultures of two edible species *Tricholoma equestre* and *Xerocomus badius* (2).

In the present study, we analyzed fruiting bodies of another species edible after appropriate preparation – *Armillaria mellea* (Vahl.) P. Kumm. (Honey mushroom). This species is common in deciduous, coniferous and mixed forests on all continents. In moderate climatic zone, the fruiting bodies are usually formed from July to November, the most abundantly in October. The species *Armillaria mellea* can occur in a saprophytic form developing on stumps, a parasitic form infecting roots of deciduous and coniferous trees or in a symbiotic form living in a mycorrhizal association with the orchid species

Gastrodia elata occurring in Asia, Australia and New Zealand.

Chemical composition of the *Armillaria mellea* fruiting bodies is largely known (3). The fruiting bodies contain pharmaceutically and consumptionally valuable groups of metabolites, including carbohydrates (4), sterols (5), sphingolipids (6), fatty acids (7), sesquiterpenoids (8), enzymes (9, 10), peptides (11) and metals (12, 13). Certain compounds possess potential antibacterial, fibrinolytic and anticancer properties and capability to protect brain (14) and bone marrow cells (15). Indole compounds in fruiting bodies of this species have not been investigated so far. Knowledge of this metabolite group seems to be important not only from theoretical but also pharmaceutical and toxicological perspective.

Analysis of indole compounds in extracts of fruiting bodies of the previously studied species *Tricholoma equestre* and *Xerocomus badius* and in mycelia from *in vitro* cultures was performed using the HPLC method described by Kysilka (16) and modified by Muszyńska et al. (2). Muszyńska et al.

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(1) developed also an original method for determination of this group of compounds, namely TLC analysis with densitometric detection and used it for analysis of *Lactarius deterrimus* extracts. The aim of the present studies was to validate the method of determination of tryptamine and serotonin by TLC analysis with densitometric detection and to use it for analysis of extracts from *Armillaria mellea* fruiting bodies. Other indole compounds, whose method of determination had been validated previously (1), were currently examined by the TLC analysis with densitometric detection.

EXPERIMENTAL

Apparatus

Linomat IV sample applicator and TLC Scanner 3 densitometer with wincats software (Camag, (Muttenez, Switzerland))

Standard substances and other chemicals

The following standard substances were used: tryptamine, L-tryptophan, 5-OH-tryptophan, serotonin, indole, melatonin, indolilacetic acid, indoleacetonitrile, indoloacetamide, kynurenine sulfate, kynurenic acid (Sigma-Aldrich). Other chemicals: ammonia – 25% solution, ethanol, petroleum ether and isopropanol all analytical grade, were purchased from Polish Reagents Company (POCh Gliwice),

Origin of fruiting bodies

The studies were conducted on *Armillaria mellea* (Vahl.) P. Kumm. fruiting bodies collected in mixed forests in southern Poland (Tunel near Kraków) in September 2008 (deposited in the Department of Pharmaceutical Botany, Jagiellonian University, Collegium Medicum, Kraków, Poland) .

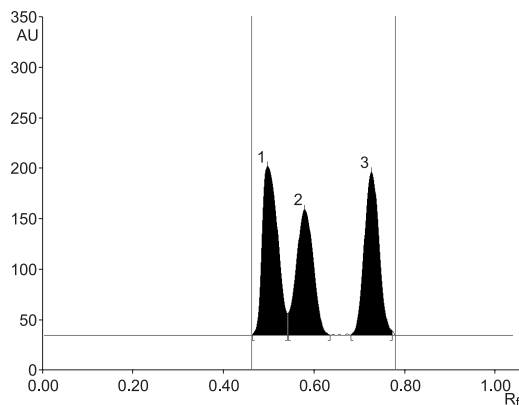


Figure 1. A densitogram of chromatogram after development; 1 – L-tryptophan, 2 – serotonin and 3 – tryptamine

Extraction and sample preparation

Lyophilized fruiting bodies (approximately 71 g) were extracted in a percolator with petroleum ether in order to remove oil fraction according to the procedure developed in our laboratory (2). Then, the material was dried and extracted with methanol (10 portions/300 mL) in a percolator for 24 h. The extract was concentrated to 42.5 mL by distillation in a vacuum evaporator under reduced pressure at 40°C. To remove the rest of lipids, the concentrated extract was frozen. For the next stage of purification of the extract, we used preparative TLC method on aluminum-backed silica gel 60 plates (Merck, Art. No 1.05554.0001), on which 3 mL of the extract was applied and chromatograms were developed in mobile phase: isopropanol : 25% NH_3 : water (8:1:1, v/v/v). Spots were identified at $\lambda = 254$ nm. Five fractions were obtained and analyzed by the TLC method with densitometric detection.

TLC analysis with densitometric detection

Solutions of standard substances, i.e., three compounds discovered in the extracts under study in preliminary experiments, were prepared. The solutions were prepared by dissolving the substances in methanol at the given concentrations: tryptamine (0.8800, 0.4400, 0.2200, 0.1100, 0.0550 and 0.0275 mg/mL), serotonin (0.5300, 0.2650, 0.1325, 0.0663, 0.0331 and 0.0166 mg/mL) and tryptophan, which was validated earlier (0.4400 mg/mL)

Separation conditions for tryptamine, L-tryptophan and serotonin were established. The chosen mobile phase was composed of: isopropanol : 25% NH_3 : water (8:1:1, v/v/v) and allowed to obtain a good separation of the substances under analysis: $R_F \sim 0.50$ for L-tryptophan, $R_F \sim 0.58$ for serotonin and $R_F \sim 0.73$ for tryptamine (Fig. 1). Spots on chromatograms were detected densitometrically at $\lambda = 280$ nm.

The method that has already been validated for L-tryptophan was chosen for the study. Conditions of determination of tryptamine and serotonin were validated by evaluation of accuracy, precision, linearity, limit of detection and limit of quantitation.

Accuracy

Accuracy of the method was characterized by assessment of percent recovery for serotonin and tryptamine. The test was performed by addition of an exactly known dose of the standard substance amounting to 80–120% of the declared content. The determination was performed before the addition of the standard substance and thereafter (Tab. 1).

Table 1. Validation parameters of the tryptamine and serotonin determination method.

Substance	R _F	Recovery [%]	Precision [mm ²]	LOD [μg/spot]	LOQ [μg/spot]	Linearity
Serotonin	~ 0.58	96.1 95.2 96.4 95.3 97.5 $\bar{x} = 96.10$ $S_x = 0.930$ $t_{95\%} \pm 1.064$ RSD = 0.97%	5045.7 4936.7 5092.1 5012.3 4982.7 $\bar{x} = 5013.9$ $S_x = 59.29$ $t_{95\%} = \pm 67.43$ RSD=1.18%	0.126	0.381	$p = 16056.8 \times c + 483.6$ $r = 0.99479$ $S_y = 204.09$
Tryptamine	~ 0.73	95.3 97.3 97.8 95.9 98.2 $\bar{x} = 96.90$ $S_x = 1.247$ $t_{95\%} = \pm 1.418$ RSD = 1.29%	4293.1 4295.7 4186.2 4210.1 4192.1 $\bar{x} = 4235.4$ $S_x = 54.55$ $t_{95\%} = \pm 62.03$ RSD = 1.29%	0.171	0.518	$p = 5503.6 \times c + 532.2$ $r = 0.99749$ $S_y = 142.57$

\bar{x} – mean value for $n = 5$, S_x – standard deviation, $t_{95\%}$ – confidence interval at 95%, RSD – variation coefficient, p – area [mm²], c – concentration [mg/mL]

Table 2. Contents of the compounds under study in the extracts of *Armillaria mellea* fruiting bodies (in mg/100 g d.w.).

Tryptamine	L-tryptophan	Serotonin
2.737	4.453	2.206
2.735	4.459	2.201
2.748	4.477	2.203
2.744	4.476	2.217
2.735	4.470	2.208
$\bar{x} = 2.740$	$\bar{x} = 4.467$	$\bar{x} = 2.207$
$S_x = 0.0059$	$S_x = 0.0106$	$S_x = 0.0062$
$t_{95\%} = \pm 0.0073$	$t_{95\%} = \pm 0.0132$	$t_{95\%} = \pm 0.0077$
RSD = 0.22	RSD = 0.24	RSD = 0.28

\bar{x} – mean value for $n = 5$, S_x – standard deviation, $t_{95\%}$ – confidence interval at 95%, RSD – variation coefficient

Precision

Precision was determined using model solutions prepared by dissolution of the substances under analysis in methanol. Five repetitions of each solution were analyzed (Tab. 1).

Linearity

Linearity was tested for the relationship between peak area (in mm²) vs. concentration (in mg/mL). Linearity was evaluated by the calculation of linear regression equations and correlation coefficients (r).

Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection and limit of quantification were calculated based on standard deviation and

slope using equations: $LOD = 3.3 S_y/a$, $LOQ = 10 S_y/a$, where: S_y = standard error of estimation, a = slope (Tab. 1)

Analysis of extracts from fruiting bodies

Standard solutions (3 μL) and fractions of test extracts (5 μL) were loaded on 10 cm × 10 cm TLC plates (coated with silica gel with fluorizing agent F₂₅₄, (Merck, Art. No 1.05554.0001) as bands 1 cm long by means of sample applicator. Chromatograms were developed to a height of 9.5 cm in a chromatographic chamber saturated with the mobile phase and were dried at room temperature. Densitometric analysis was performed at a wavelength 280 nm using Camag TLC- Scanner 3 densitometer with wincats software. The components were identified based on R_F value for spots obtained

with standard substances and test extracts. The components were quantified based on peak areas.

The method developed in the present study was used for analysis of fractions obtained by preparative chromatography, which were preliminarily identified under UV lamp. The studies aimed to detect L-tryptophan, tryptamine and serotonin, confirmed their presence in some fractions from preparative chromatography. Detailed data on the contents of the components under study are presented in Table 2.

RESULTS AND DISCUSSION

Validation of the method for determination of serotonin and tryptamine

The conditions of thin layer chromatography with densitometric detection in UV range allow for identification and concomitant quantitation of tryptamine, tryptophan and serotonin in methanolic extracts of *Armillaria mellea* fruiting bodies. Qualitative and quantitative analysis of these components require their prior preliminary isolation according to the above-described procedure of analyzed preparation by extraction and subsequent extract purification by preparative TLC. The components of the extracts under study were analyzed using the mobile phase composed of isopropanol : 25% NH_3 : water (8:1:1, v/v/v), which enabled separation of the substances under study as demonstrated with standard solutions (Fig. 1). The differences in retention coefficient were significant, which facilitated identification of individual components. Densitometric measurements at $\lambda = 280$ nm allow for recording of peaks directly from chromatograms. The peaks are well resolved and easy for quantitative evaluation (Fig. 1).

Validation parameters for the method of serotonin and tryptamine determination (Tab. 1) are comparable with validation parameters for tryptophan (1). Validation results presented in Table 1 indicate that the proposed method is characterized by a high sensitivity; LOD for serotonin is 0.126 mg, and 0.180 mg for tryptamine (0.075 mg for L-tryptophan); LOQ was estimated at 0.381 mg, 0.518 mg and 0.226 mg, respectively. Recovery of the components under study amounts to: 96.10%, 96.90% and 99.09%, respectively. The method is also characterized by high precision, RSD for the substances under analysis equals to: 1.18%, 1.29% and 1.40%, respectively.

Linearity of the concentration vs. peak area curve for L-tryptophan, tryptamine and serotonin was preserved in a wide concentration range: from

0.0165 mg/mL to 0.5300 mg/mL for serotonin, from 0.0275 mg/mL to 0.8800 mg/mL for tryptamine and from 0.0369 mg/mL to 0.5900 mg/mL for L-tryptophan.

Application of the chromatographic-densitometric method for analysis of the extracts allowed for a precise, relatively easy and quick determination of the compounds under study.

Quantitative analysis of the contents of indole compounds

Analysis of methanolic extracts from *Armillaria mellea* fruiting bodies by TLC method with densitometric detection demonstrated the presence of three main metabolites: L-tryptophan, tryptamine and serotonin.

We demonstrated previously that the extracts of fruiting bodies of other *Basidiomycota* species, contained a richer qualitative composition of this group of compounds because five different compounds were identified in each *Lactarius deterrimus* and *Xerocomus badius* (1, 2) and four compounds were detected in *Tricholoma equestre* fruiting bodies (2). Only L- tryptophan and tryptamine were common to fruiting bodies of *Armillaria mellea* and three previously studied species of mushrooms. Serotonin was found in the currently investigated *Armillaria mellea* and in two species *Tricholoma equestre* and *Xerocomus badius* examined earlier.

Armillaria mellea fruiting bodies contained significant amounts of the metabolites: 4.467 mg/100 g d.w. for L-tryptophan, 2.740 mg/100 g d.w. for tryptamine and 2.207 mg/100 g d.w. for serotonin. These contents were less diverse than those determined in *Lactarius deterrimus* fruiting bodies which ranged from 0.190 (melatonin) to 2.728 mg/100 g d.w. (tryptamine) (1).

Tryptophan contents in the extracts of *Armillaria mellea* fruiting bodies under study were many times higher than in previously investigated *Lactarius deterrimus* (0.235 mg/100 g d.w.). High concentration of this compound in food products, including fruiting bodies of mushrooms is not safe because tryptophan ingested as an exogenous amino acid at too high doses can damage the nervous system (17) and can contribute to the induction of urinary bladder cancer (18). Moreover, this amino acid is a precursor of all other indole compounds showing hormonal activity as tissue hormones.

Tryptamine contents in the currently studied extracts of *Armillaria mellea* fruiting bodies were marked and almost identical as in *Lactarius deterrimus* (2.728 mg/100 g d.w.). From pharmacological and toxicological point of view, tryptamine is not a

safe compound. It can interact with MAO inhibitors and can even cause deadly poisoning in patients taking these drugs. Tryptamine and its derivatives are psychoactive substances showing agonistic activity at 5-HT₂ receptors (18).

Serotonin contents in *Armillaria mellea* fruiting bodies were high. The contents of this compound determined with another method in fruiting bodies of *Tricholoma equestre* and *Xerocomus badius* were significantly lower, amounting to 0.524 and 0.182 mg/100 g d.w., respectively. Although serotonin has recently been proven to possess beneficial biological properties, like antioxidant action and therapeutic potential in Alzheimer's disease (19, 20), the high content of this compound, fulfilling the role of tissue hormone in human organism, in the fruiting bodies under study (2.207 mg/100 g d.w.) calls for caution when this mushroom is eaten in larger amounts. This caution is required also due to the proven concomitant presence of two other indole compounds, tryptamine and tryptophan, in these fruiting bodies.

CONCLUSION

The present studies have demonstrated for the first time that extracts of *Armillaria mellea* fruiting bodies contain L-tryptophan, tryptamine and serotonin. In this work, a method of determination of tryptamine and serotonin (TLC analysis with densitometric detection) was developed and validated and was successfully used for mycochemical analysis of extracts from fruiting bodies. Tryptophan was determined using a previously developed and validated original method (also TLC analysis with densitometric detection), which was evaluated in earlier mycochemical studies of *Lactarius deterrimus* fruiting bodies.

High contents of L-tryptophan, tryptamine and serotonin, shown in the present study, justify further detailed studies on the accumulation of indole compounds in this species.

REFERENCES

1. Muszyńska B., Maślanka A., Sułkowska-Ziaja K., Krzek J.: J. Planar Chromatogr. Modern TLC 20, 55 (2007).
2. Muszyńska B., Sułkowska-Ziaja K., Ekiert H.: Pharmazie 64, 479 (2007).
3. Muszyńska B., Sułkowska-Ziaja K., Wołkowska M., Ekiert H.: J. Med. Mushrooms (submitted).
4. Chen X.M., Guo S.X., Wang Q.Y., Xiao P.G.: Quel. Zhongguo Zhong Yao Za Zhi 26, 381 (2001).
5. Rajewska J., Bałasińska B.: Postępy Hig. Med. Doświad. 58, 352 (2004).
6. Gao J.,M., Yang X.: Fitoterapia 72, 858 (2001).
7. Arnone A., Cardillo R., Gianluca N., Valdo Meille S.: Mycol. Res. 110, 414 (2006).
8. Yang J.S., Su Y.L., Wang Y.L., Feng X.Z., Yu D.Q., Liang X.T.: Planta Med. 55, 479 (1989).
9. Healy V., O' Connell J., McCarthy T.,V., Doonan S.: Biochem. Biophys. Res. Commun. 262, 60 (1999).
10. Lewis W.G., Basford J.M., Walton P.L.: Biochim. Biophys. Acta 522, 551 (1978).
11. Conlon J.,M., Eriksson B., Grimelius L., Oberg K., Thims L.: Biochem. J. 248, 123 (1987).
12. Falandysz J., Sicińska B., Bona H., Kohonke D.: Bromatol. Chem. Toksykol. 25, 171 (1992).
13. Kalač, P., Svoboda L., Havlíčková B.: Energy Edu. Sci. Technol. 13, 313 (2004).
14. Lee S., Kim J., Kim J., Sapkota, K.: Protein Expr. Purif. 43, 10 (2005).
15. YP., Wu K.F., Liu Y.: Zhongguo Zhong Yao Za Zhi 30, 283 (2005).
16. Kysilka R., Wurst M. J. Chromatogr. 320, 414 (1985).
17. Svrček M., Vančura B.: Houby. p. 162, Artia, Praha 1983.
18. Stone T. W., Mackay G. M., Forrest C. M., Clark C. J., Darlington L. G.: Clin. Chem. Lab. Med. 41, 852 (2003).
19. Turek M., Łodyga-Chruścińska E.: Food Chem. Biotechnol. 72, 73 (2008).
20. Ouchi Y., Yoshikawa E., Futatsubachi M., Yagi S., Ueki T., Nakamura K.: J. Nucl. Med. 50, 1260 (2009).

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